Universidade de Lisboa
Faculdade de Ciências
Departamento de Biologia Vegetal

INTERPLAY BETWEEN INTESTINAL MICROBIOTA AND INNATE LYMPHOID CELLS

Hélder Manuel Piedade Ribeiro

Dissertação

MESTRADO EM MICROBIOLOGIA APLICADA

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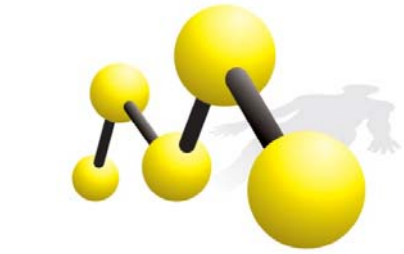

INSTITUTO DE MEDICINA MOLECULAR

FACULDADE DE MEDICINA DA UNIVERSIDADE DE LISBOA

INTERPLAY BETWEEN INTESTINAL MICROBIOTA AND INNATE LYMPHOID CELLS

Hélder Manuel Piedade Ribeiro

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This thesis was fully performed at Instituto de Medicina Molecular under the direct supervision of Dr. Henrique Veiga-Fernandes in the scope of the Master in Applied Microbiology of the Faculty of Sciences of the University of Lisboa.

I. RESUMO

A interação entre a microbiota intestinal e o sistema imunitário é essencial para a manutenção da homeostasia intestinal. No entanto, desequilíbrios na relação hospedeiro-microrganismos levam a doenças inflamatórias que se revelam um problema de saúde pública. Assim, a composição bacteriana do intestino é importante para preservar a barreira intestinal. As bactérias comensais que habitam o muco do cólon desempenham um papel mais importante na estimulação de respostas imunes reguladoras em comparação com bactérias luminais, uma vez que vivem perto das células epiteliais intestinais.

As células linfóides inatas (ILC) são uma família emergente de células efetoras, abundantemente presentes nas mucosas. O Grupo 3 das ILC (ILC3) produz citoquinas pró-inflamatórias e regula a homeostasia da mucosa, a defesa anti-microbiana e as respostas imunitárias adaptativas. Neste estudo utilizámos ratinhos com mutações de perda e ganho de função de recetores neuroreguladores, para definir o papel destas moléculas na função das ILC3, na homeostasia e defesa da mucosa intestinal e na manutenção da ecologia microbiana intestinal.

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A análise de ratinhos adultos revelou que as ILC3 expressam um recetor neuroregulador, a tirosina cinase RET, que é uma molécula crítica para o desenvolvimento e manutenção de células nervosas, entre outras. Surpreendentemente, os sinais RET controlam a expressão de IL-22, uma citocina muito importante para a homeostasia do intestino. Por conseguinte, verificámos que a deficiência em *Ret* está associada a um decréscimo da expressão de péptidos antimicrobianos, mucinas, e beta-defensinas em células epiteliais entéricas.

Descobrimos que os sinais do recetor RET são críticos para a defesa do intestino, durante a colite induzida por DSS. Enquanto os ratinhos *Rorc*-Cre/*Ret*$^{fl/fl}$, que têm uma deficiência em *Ret* especificamente em RORγt^{pos} ILC3s, têm uma redução de IL-22^{pos} ILC3, aumento de inflamação e de translocação bacteriana a partir do tracto gastro-intestinal, mutantes com ganho de função de RET (*Ret*^{MEN2B}) estão altamente protegidos comparando com controlos selvagens da mesma ninhada. Surpreendentemente, descobrimos que os

ratinhos *Rorc*-Cre/*Ret*$^{fl/fl}$ possuem uma microbiota alterada, nomeadamente, com o aumento dos níveis de Proteobacteria e Bacteroidetes. Encontrámos também, durante o tratamento com DSS, uma diminuição na carga de Firmicutes, um dos filos com maior número de bactérias intestinais e, normalmente faz parte da flora intestinal saudável.

Os nossos resultados mostram uma relação fundamental entre fatores neurotroificos,ILC3 e células epiteliais na homeostasia da mucosa e defesa intestinal. Assim, este trabalho elucida novos aspetos da biologia das ILC3, revelando novos mecanismos que poderão ser explorados como alvos terapêuticos em doenças infeciosas e inflamatórias com grande impacto na saúde pública.

Palavras-chave: microbiota; intestino; sistema imunitário do intestino; células linfóides inatas; recetor tirosina cinase RET.

II. SUMMARY

The interplay between the intestinal microbiota and the immune system is essential for intestinal homeostasis. However, inadequate host-microbe relationships lead to inflammatory diseases that are major public health concerns. Hence, the composition of commensal bacteria is important to preserve colonic health. The commensal bacteria that inhabit the colonic mucus have been suggested to play a critical role in stimulating regulatory immune responses when compared to their luminal counterparts, since the former live close to the intestinal epithelial cells.

Innate lymphoid cells (ILC) are an emergent family of effectors cells, abundantly present at mucosal sites. Group 3 ILC (ILC3) produces pro-inflammatory cytokines and regulates mucosal homeostasis, anti-microbial defence and adaptive immune responses. In this study, we interrogated the role of neuroregulatory receptors in ILC3 function, mucosal homeostasis, gut defence and microbial ecology. To this end, we employed ILC3-specific loss and gain of function mutant mice for the neurotrophic factor receptor RETS.

Analysis of adult ILC3 revealed that they uniquely express the neurotrophic factor receptor RET, which is a critical molecule for the development and maintenance of neuronal cells, amongst others. Herein, we found that *Ret* deficiency led to decreased innate IL-22 and deficit of antimicrobial peptides, mucins, and beta-defensins in enteric epithelial cells.

We found that RET signals are critical keepers of gut defence, in DSS-induced colitis. While *Rorc*-Cre/*Ret*$^{fl/fl}$ mice, exhibiting *Ret* ablation in RORγt expressing ILC3s, had reduced IL-22^{pos} ILC3, increased inflammation and bacterial translocation from the gastro-intestinal tract, RET gain-of function mutants (*Ret*^{MEN2B}) were highly protected over their WT littermate controls. Strikingly, we found that *Rorc*-Cre/*Ret*$^{fl/fl}$ mice have altered microbiota, notably increased levels of Proteobacteria and Bacteroidetes. Noteworthy, *Rorc*-Cre/*Ret*$^{fl/fl}$ also had a decrease load of Firmicutes during enteric inflammation. This phylum is one of the most abundant intestinal entities and normal levels of these bacteria are characteristic of a healthy flora.

Our results show that the interplay between neurotrophic factors, ILC3 and epithelial cells are critical to mucosal homeostasis and defence. Thus, our brings novel insights to ILC biology and microbiota, paving the way for new therapeutic targets in infectious and inflammatory diseases that are major Public Health concerns.

Key words: microbiota; intestine; enteric immune system; innate lymphoid cells; tyrosine kinase receptor RET.

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IV. INTRODUCTION

1. GENERAL ASPECTS

After birth the mammalian body is colonised by aerobic and anaerobic microorganisms, including viruses, bacteria, and eukaryotic parasites[1]. The first exposure to microbes usually occurs at birth as a result of maternal/offspring contact and from then on the microbial communities are shaped by the genetics and the environment. These communities are present at all body surfaces, such as the skin, the respiratory tract and the intestine, and are referred to as the "microbiota"[1,2,3].

The majority of this microbiota lives in the distal region of the gastrointestinal tract (GIT), and it has been estimated that the intestinal mucosa homes up to 10^{13} to 10^{14} microorganisms[4]. Thus, bacteria are ten times more numerous than the total number of human cells in the body. Moreover, the microbiome, the collective genome of the microbiota, contains at least 100-fold more genes than the human genome[5,6].

The consequence of the microbial colonization is an intimate relationship between host and microbes. This tightly regulated interplay has co-evolved for mutually beneficial outcomes and it has been show to regulate the development and function of cells from the host immune system and to enhance the intestinal barrier[7].

In health, the microbiota provides protection against pathogens, induction of immune regulatory functions, nutrient processing and several metabolic functions[3]. More specifically, the enteric microbiota allows for energy extraction from otherwise indigestible dietary polysaccharides and also leads to the production of essential nutrients, such as short-chain fatty acids (SCFA), vitamins (e.g. vitamin K, vitamin B12 and folic acid) and amino acids[8]. In turn, the intestine provides a nutrient-rich environment in which the microbiota establishes a diverse, but yet extremely stable and resilient ecosystem[8].

2. DIVERSITY OF INTESTINAL MICROBIOTA

Despite the important role played by the intestinal microbiota, how the microbial composition is determined remains poorly understood. Most intestinal bacteria are located in the lumen, in the mucus covering the epithelium, in the crypt spaces and in various cells lining the epithelium, such as epithelial cells[5,9]. It has been estimated that circa 400 bacterial species compose the mammals' intestinal microbiota[8,9]. However, further knowledge on the exact composition of the microbiota has been hampered by microbial culture limitations since only a small fraction (20-40%) of the species is accessible through culture-based techniques[9,10]. This is partly due to strict oxygen supplies of some species and the fact that some other species only growth in a high level of inter-microbe mutualistic interactions[10].

Nevertheless, the more recent developments of culture-independent molecular methods based on 5S and 16S ribosomal RNA (rRNA) gene sequence have expanded our knowledge on the composition of the intestinal microbiota[11,12]. 16S rRNA sequence-based methods revealed that two bacterial phyla: Bacteroidetes and Firmicutes, comprise more than 90% of the known phylogenetic categories and dominate the distal gut microbiota[13]. Nevertheless, there is a substantial diversity of the gut microbiome between healthy individuals[13].

2.1. HEALTHY MICROBIAL COMMUNITIES

The GIT colonization begins immediately at birth and the microbiota differs between infants and adults[10]. The vast majority of microbial cells in the human GIT are bacteria, including aerobic, anaerobic and facultative anaerobic bacteria[10,14].

Bacterial composition varies along the intestinal tract, as each species of bacteria colonises a discrete niche. While anaerobic microorganisms are absent in the stomach, these are predominant in the distal colon[14]. Anaerobic bacteria progressively increase from the proximal to distal regions, and 99% of the residents

located in the large intestine are anaerobes[15]. However, facultative anaerobes have a tendency to associate along the epithelial layer where oxygen diffusing from tissues is available; this is the case for *E. coli* and possibly also for other organisms[9].

The major phyla among intestinal eubacteria in healthy individuals are Gram-negative Proteobacteria and Bacteroidetes, and Gram-positive Firmicutes, such as *Clostridiales* and *Lactobacillales*[3]. The phylum Bacteroidetes consists of three classes including the well studied genera *Bacteroides* and *Prevotella*. Firmicutes is currently the largest known bacterial phylum, containing more than 200 genera[3,10]. The methanogens, such as, *Methanobrevibacter smithii* and *Methanosphaera stadtmanae,* are the predominant intestinal archaea, that obtain energy from hydrogen molecules[3,16]. Finally, the fungal phyla *Ascomycota* (which includes the genera *Candida* and *Saccharomyces*) and *Basidiomycota* are the major fungal entities in the GIT (Figure 1a)[8].

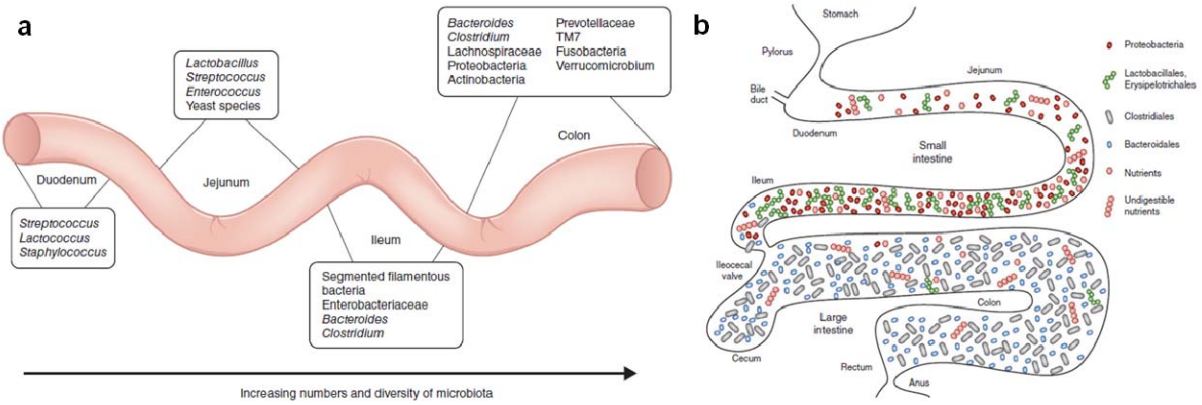

Figure 1 – Distribution of predominant bacteria in the GIT. The amount and composition of microbial species varies along the intestinal tract. **a.** Phyla, families and genera of the microbiota. The main bacterial phyla are represented in the mammalian gut microbiota: Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, Verrucomicrobia, Cyanobacteria, Fusobacteria, Spirochaetes and TM7. Eukaryotic and Archaeal microorganisms also can inhabit the intestine in low frequency. **b.** The small intestine is rich in nutrients used by both the host and the microbes. The small intestine is dominated by Proteobacteria spp. (mainly Enterobacteria), Lactobacillales and Erysipelotrichales (especially *Turicibacter* spp.). In other hand, the large intestine, rich in host-indigestible fibers, is dominated by Bacteroidetes and Clostridia (Adapted from REF.[3,16])

The small intestine, where Proteobacteria and Lactobacillales are highly abundant, is rich in monosaccharides and disaccharides as well as amino acids[10]. In the distal small intestine, including the terminal ileum, bacterial growth is substantially altered due the fact that simple sugars are absorbed at this site by host cells[16] (Figure 1b).

The colon is the main site for fermentation and, due to its slow transit rate and rich nutritional environment; the bacterial density is very high, with about 10^{12} cells/g of luminal content[17,18]. The large intestine ecosystem is dominated by Firmicutes (mainly *Clostridium* cluster XIV) and Bacteroidetes[17]. Proteobacteria, such as *Escherichia coli*, cannot digest polysaccharides and, consequently, cannot use complex carbohydrates as an energy source[16,19]. In contrast, *Bacteroides* and *Clostridiales* are the dominant populations in the large intestine and have enzymes that can break down host-indigestible polysaccharides, including fibers and mucins. Thus, the distribution of nutrients in the intestine is a major driver of the microbiota composition in the gut[16].

2.2. ALTERED MICROBIAL COMMUNITY

Several studies have shown differences in the composition of microbiota between healthy individuals and patients with intestinal diseases[20]. The interplay between commensal bacteria and the host is tightly regulated and plays a central role in the intestinal homeostasis[21]. However, perturbation of this equilibrium has been linked to intestinal inflammation, which is a main feature of devastating diseases, including Ulcerative colitis (UC) and Crohn's disease (CD), the two major inflammatory bowel diseases (IBD)[22]. As such, dysbiosis of the gut microbiota has been associated with these severe pathologies. Dysbiosis, referred to as the disrupted microbiota equilibrium, results in an altered microbiota metabolism and overgrowth of pathobiont bacteria, which makes the host more susceptible to multiple pathological conditions (Figure 2)[23,24].

UC is an idiopathic IBD, which is characterised by chronic inflammation of the colonic mucosa and may be restricted to the distal colon and rectum[25]. The classical symptoms are diarrhea with passage of blood, mucus or both, occasional abdominal cramping, pain as well as systemic symptoms of fever and in severe cases weight loss[25,26]. Frank et al. have demonstrated that the faecal and mucosal amount of Firmicutes was significantly lower in UC patients than in healthy subjects[27]. The

etiology of IBD is still unknown but recent evidence indicates that it involves a multifaceted interplay between genetic, environmental and microbial factors[28].

Pathogens can also proliferate and promote inflammation if the resident bacterial community is disrupted by treatment with antibiotics or if recipient mice have low-complexity microbiota[29]. For example, in mice previously treated with antibiotic, inoculation of virulent *E. coli,* which is normally suppressed by commensal bacteria, allows this specie to accumulate and disseminate systemically (Figure 2)[23,30].

Two well established mice models of IBD are commonly used in the laboratory: chemical (Dextran Sodium Sulphate, DSS) and infectious (*Citrobacter rodentium*)[31]. These models are characterised by a vigorous inflammatory response in the colon mucosa and can drive a shift in microbial diversity, with a replacement of the resident Firmicutes with Proteobacteria [1,8,16].

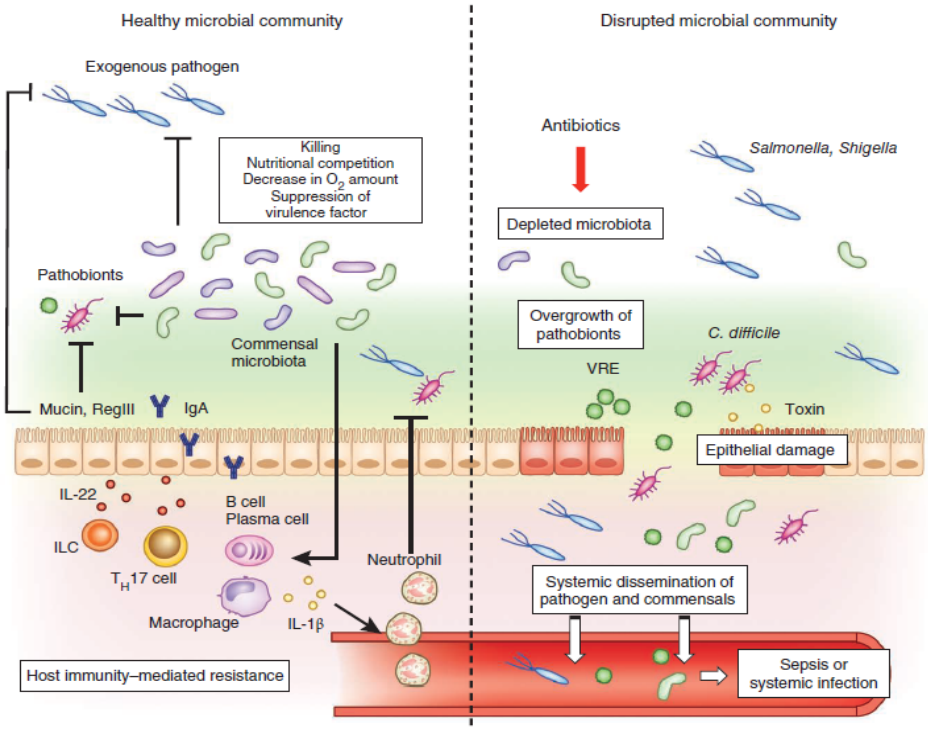

Figure 2 – The resident bacteria live in intestinal niches in the healthy gut and prevent colonization by exogenous pathogens and pathobionts through multiple mechanisms. The microbiota produces bacteriocins and short-chain fatty acids, to prevent colonization by exogenous pathogens and pathobionts. Commensal microbiota stimulates production of antimicrobial molecules, such as RegIIIβ and γ (RegIII), and regulating secretion of IgA. Differentiation and/or activation of TH17 cells and innate lymphoid cells (ILCs), promoted by commensal bacteria, control both commensals and pathogens through secreted cytokines, such as IL-22. Antibiotic treatment or other environmental issue that disrupt the commensal microbial community result in reduced resistance to colonization by pathogens (for example, *Salmonella* and *S. flexneri*). Disruption allows the outgrowth of indigenous pathobionts, like *C. difficile* and Vancomicin-Resistant Enterococci (VRE) that have the potential to propagate systemically and induce septic shock and/or systemic organ infection(Adapted from REF.[16])

3. INTESTINE

Homeostasis between the intestinal microbiota and the immune system ensures vital functions of the organism, such as efficient energy and metabolite extraction from food, protection from pathogenic and deviating symbiotic microbes, and degradation of xenobiotics and maintenance of a robust epithelial barrier[32].

The small intestine and the large intestine are physiologically distinct sites. While nutrient absorption occurs in the small intestine, the main roles of the large intestine are food storage, absorption of water and electrolytes and energy extraction from indigested carbohydrates[33]. This capacity to process otherwise indigestible components influences the gut environment and the host[34].

The small intestine epithelium is a single layer of cells derived from multipotent stem cells located within the intestinal crypts. Enteric epithelial cells are responsible for antimicrobial peptide production and maintenance of the intestinal mucus layer[35].

Anatomically, the large intestine is composed by the caecum, ascending colon, transverse colon, descending colon, sigmoid colon and rectum[34,36]. The colon wall structure includes several tissue layers, i.e., mucosa, submucosa, muscularis externa, and serosa. The mucosa consists of a mucus layer, single layer of epithelium, the lamina propria, and a thin muscle layer (muscularis mucosae)[36]. The intestinal epithelium is a monolayer of epithelial cells that includes M cells, goblet cells, Paneth cells, and columnar epithelial cells, all of which originate from crypt stem cells (Figure 3)[34].

3.1. EPITHELIAL CELL BARRIER

Mucosal surfaces represent the first line of defence against invading pathogens. The intestinal epithelial cell (IEC) layer provides a dynamic physical barrier that separates commensal bacteria from the lamina propria (LP)[36]. The abundance and proximity of these microbes to the host epithelium represents a major challenge, as the host must

control the potential for opportunistic disruption of the intestinal barrier, as well as invasion of the IEC layer[36]. Innate immune strategies include the use of a mucus layer, antimicrobial peptides (AMPs) and innate lymphoid cells (ILCs) that act on a concerted manner to confine much of the bacterial communities to the lumen of the intestinal tract (Figure 2). The mucus layer is actively modulated by infection and is regulated by innate and adaptive immune cells[3].

The colonic mucus consists of two layers rich in gel-forming mucins: an outer glycan-rich layer in which the commensal bacteria reside and a dense inner layer, tightly associated with the epithelium and mostly devoid of bacteria. The commensal bacteria colonizing the outer mucosal layer have evolved diverse microbial characteristics, which contribute to a specific selected mucosal community. Bacteria developed adhesion molecules to bind to the mucus, fimbriaes, extracellular mucus-binding protein, as well as the ability to gain nutrients from the host-derived mucins by synthesizing mucin-degrading enzymes. (Figure 2-3)[37,38].

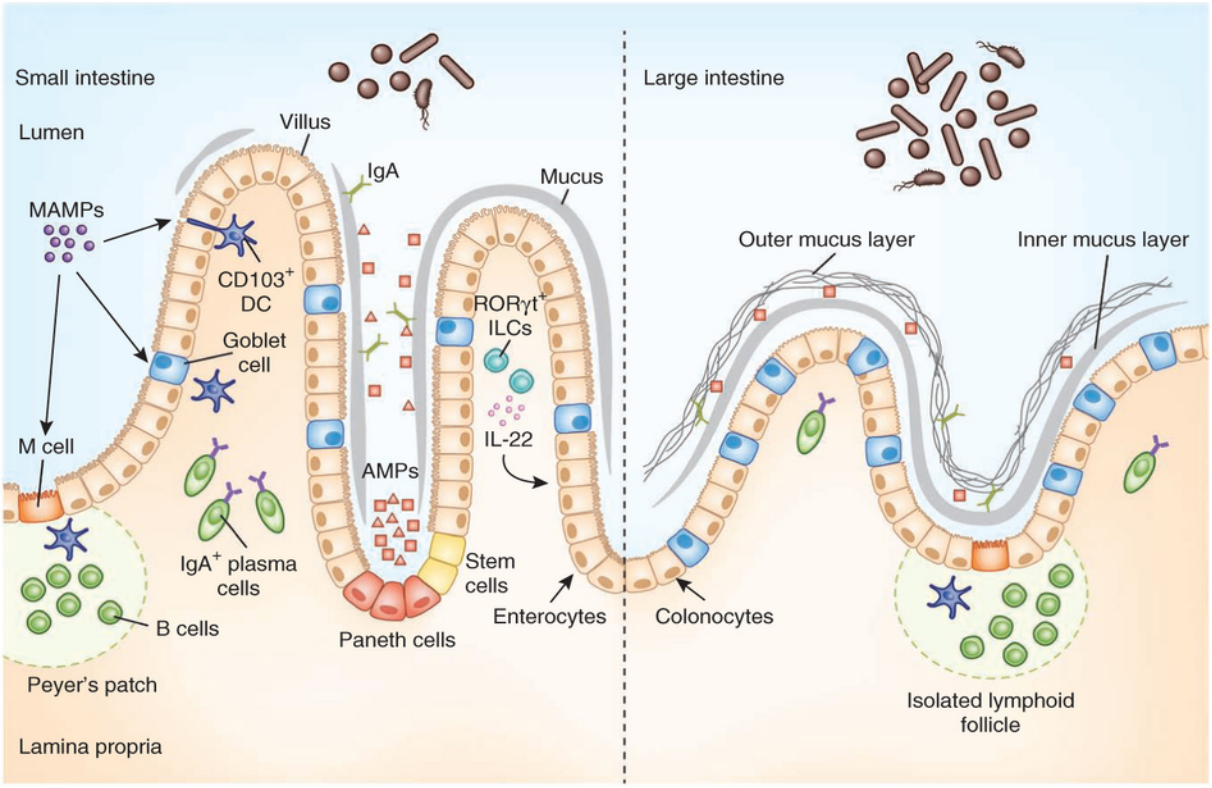

Figure 3 – A single layer of enterocytes or colonocytes composes the intestinal epithelium, which protects the integrity of the epithelial barrier. A discontinuous thinner layer of mucus gel covers the epithelial cells that line the epithelium of the small intestine, with smaller amount of goblet cells. Paneth cells are enriched in the crypts of the small intestine, produce and secrete antimicrobial peptides. Through this barrier, sampling of microorganism-associated molecular patterns (MAMPs) can be mediated through antigen uptake by M cells and goblet cells to dendritic cells (DCs). Microbiota and your microbial signals are sensing by RORγt ILCs, which produce IL-22 to maintain IEC barrier function. Plasma cells in the lamina propria, mediated by DCs in a T cell–independent mechanism produces commensal-specific IgA. The large intestine has a thick, continuous mucus layer to compartmentalize the microbiota, taking the IgA and AMPs as secondary role. (Adapted from REF.[3])

The intestine can home a number as high as 10^{12} bacteria per gram of faeces and the mucus monolayer is a vital component to segregate the microbiota from the intestinal epithelium[36]. On the other hand, the epithelium is a physical barrier, consisting in different types of cells: goblet cells (mucin secreting cells), Paneth cells (antimicrobial peptides producers), and enterocytes or colonocytes (just colon cells)[9]. These cells differentiate from epithelial stem cells residing in the villous crypt region and are linked together along the edges of their luminal surface by tight junctions (Figure 2-3)[36].

Disruption of the epithelial barrier results in invasion by commensal and pathogenic bacteria that trigger local pro-inflammatory immune responses. Studies with mice deficient for mucin 2 (*Muc2*) demonstrated that a reduced inner mucus layer allows

direct contact of the microbiota with the epithelial layer[39]. This can explain the spontaneous colitis that develops in these mice. Thus, epithelial cells strongly mediate the interaction between the mucosal immune system and the commensal microbiota[3,9].

3.2. PATTERN-RECOGNITION RECEPTORS – PRRS

The mechanisms underlying host tolerance to commensals are not fully understood, but the ability of the host's mucosal immune system to distinguish between commensal and potentially pathogenic microbial components using a limited number of pattern-recognition receptors (PRRs) is believed to play an important role in this process[40].

PRRs are germline-encoded receptors that can recognize conserved molecular structures known as pathogen-associated molecular patterns (PAMPs) or microorganism-associated molecular patterns (MAMPs) since these structures are also found in nonpathogenic microorganisms[40,41]. PRRs are expressed by innate immune cells including macrophages and dendritic cells (DCs), by adaptive immune cells such as T cells, and by nonprofessional cells such as IECs. PRRs can be divided into: Toll-like receptors (TLRs) and nuclear oligomerization domain–like receptors (NLRs), which recognize MAMPs, including lipopolysaccharide, lipid A, peptidoglycan, flagella and microbial RNA/DNA[23].

TLR2 was shown to be a major player in gut homeostasis by exerting cytoprotective effects in intestinal epithelial cells[6]. PRR-MAMP interactions are critical in promoting mucosal barrier function, regulating the production of mucin glycoproteins, AMPs (antimicrobial peptides), IgA and IL-22 [42](Figure 3). Expression of these PRRs is critical for maintaining the intestinal microbiota homeostasis (Figure 4)[42].

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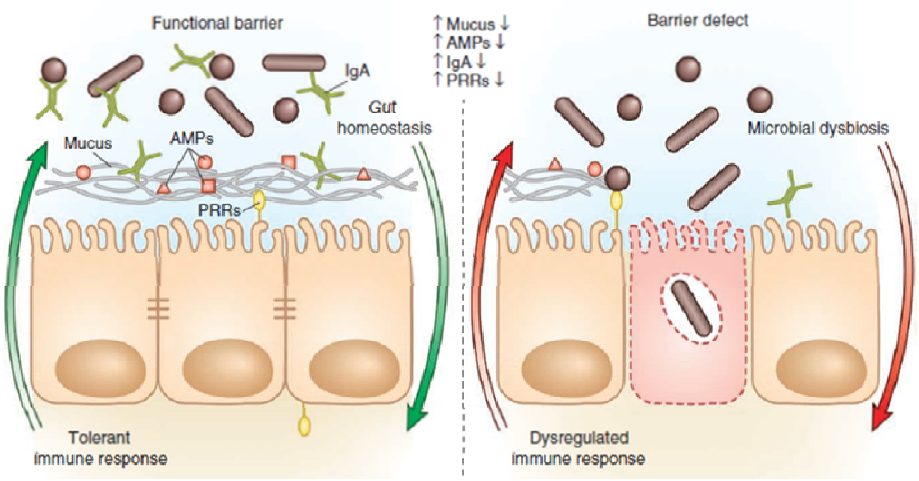

Figure 4 – Shifts in the microbiota can drive immune dysfunction. The existence of an efficient epithelial barrier, with regular amounts of PRRs, mucus, AMPs and secreted IgA, ensure intestinal homeostasis with the microbiota. (Adapted from REF.[3]).

3.3. GUT-ASSOCIATED LYMPHOID TISSUE – GALT

The intestinal microbiota directly influences host lymphoid structure development[43]. The GALT is the largest collection of immune structures and cells in the mammalian body. The GALT is a network of secondary lymphoid structures including Peyer's patches (PPs), mesenteric lymph nodes (MLNs), cryptopatches (CPs) and isolated lymphoid follicles (ILFs)[44].

Peyer's patches and MLNs develop prenatally, while CPs and ILFs develop postnatally and require signals from intestinal microbiota for their complete development and recruitment of a mature complement of immune cells, notably B cells[32]. Secondary lymphoid organ formation depends on a subset of type 3 innate lymphoid cells (ILC3) denominated lymphoid tissue inducer (LTi) cells[45].

4. INNATE LYMPHOID CELLS – ILCs

ILCs are emerging as important effectors of innate immunity and are defined by absence of recombination activating gene (*Rag*)-dependent rearranged antigen receptors; a lack of myeloid and dendritic cell phenotypical markers; and their lymphoid morphology. The prototypical ILCs are lymphoid tissue-inducer (LTi) cells, crucial for the development of CPs, PPs and iLFs; and natural killer (NK) cells[7].

ILCs require the common cytokine receptor γ-chain (γc; also know as IL-2Rγ) and the transcriptional repressor inhibitor of DNA binding 2 (ID2) for their development (Figure 5)[7].

Their strategic location in the gastrointestinal gut and their capacity to secrete large amounts of immune response mediators (cytokines) play a critical role in regulating epithelial cell responses and maintaining intestinal homeostasis. ILCs locate in the lamina propria in close contact with dendritic cells. ILCs contribute to host defence through the expression of cytokines[46].

The combined expression of lineage-specific transcription factors with discrete cytokine profiles led to the identification of three distinct ILC subsets[7]. Group 1 ILC (ILC1) includes helper ILC1[47,48,49,50] and cytotoxic NK cells and produces IFNγ (Figure 5)[7], an important stimulator of mononuclear phagocytes that promotes antimicrobial activity and protection to intracellular infections such as *Mycobacterium tuberculosis* or *Toxoplasma gondii*[51].

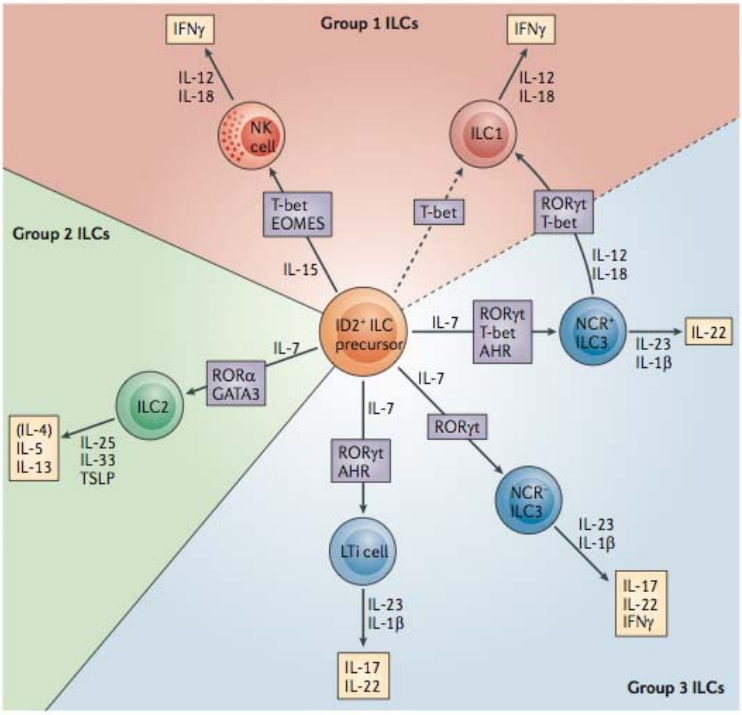

Figure 5 – Classification of ILCs on basis of their functional characteristics**.** Group 1 ILCs, NK cells and ILC1s require T-bet for their development and function and express IFNγ. Group 3 ILCs, LTi cells (LTi4 in the adult), NCR^{pos} ILC3 and NCR^{neg} ILC3s (LTi0) development highly depends on RORγt expression, as well as their function. They all express IL-22, but LTi4 cells and LTi0 express IL-17 and LTi0 also expresses IFNγ. Lastly, Group 2 ILCs, require GATA3 for their development and express typical Th2 cytokines. (Adapted from REF.[7])

ILC1 were shown to depend on TBX21, IL-7 and IL-15[7]. Group 2 ILC are RORα[52,53] and GATA3[54,55,56,57] dependent, IL-7 dependent and produce IL-5 and IL-13 (Figure 5)[7,58,59]. ILC2 have been shown to play important roles in helminth infections, asthma and allergy[52,60]. Group 3 ILC (ILC3) is RORγt and partly AhR dependent, relies on IL-7 and produces IL-17 and IL-22 (Figure 5)[7,60,61,62]. ILC3 were shown to mediate inflammatory bowel diseases[71] and to efficiently control immune responses to *Candida albicans*[63] and to attaching and effacing enteric pathogens, such as *Escherichia coli* in humans and *Citrobacter rodentium* in mice[64,65]. More recently, ILC3 were shown to be central regulators of adaptive lymphocyte responses[66,67].

4.1. GROUP 3 INNATE LYMPHOID CELLS

Lymphoid tissue inducer (LTi) cells are the prototypical member of ILC3, and play a major role in secondary lymphoid organ development in the foetus and tissue homeostasis in adult life[68]. LTi cells are mainly found at mucosal sites in adulthood and are major components of enteric cryptopatches (CP) and isolated lymphoid follicles (ILF). Some ILC3 express the natural cytotoxicity receptor (NCR) NKp46 and were named NCR^{pos} ILC3[7,49,69,70,71]. NCR^{pos} ILC3 development depends on *Notch* downstream of the transcription factor T-bet[72,73]. NCR^{pos} ILC3 express IL-22 but not IL-17. IL-22^{pos} ILC3 are critical for innate immune responses against attaching-effacing bacteria, such as *Citrobacter rodentium*[69,71,74,75]. IL-22 sustains mucosal barrier integrity and prevents damage induced by pathogens or inflammation[65].

As an example, IL-22 induces the expression of antimicrobial peptides such as S100A7, S100A8, S100A9, β-defensin-2, and β-defensin-3[76,77,78,79,80]; it promotes RegIIIβ and RegIIIγ expression from intestinal epithelial cells[64] and stimulates the production of mucins (*Muc1*, *Muc3*, *Muc10*, and *Muc13*) from goblet cells[81]. Another ILC3 subset lacks NCR expression and produces IFNγ and IL-17 in addition to IL-22 and were named NCR^{neg} ILC3[7]. NCR^{neg} ILC3 mediate pathology in innate colitis mouse models and are increased in inflammatory bowel disease (IBD) patients[82,83]. Nevertheless, the mechanisms that balance tissue protective ILC and their pathogenic counterparts remain unknown.

4.2. NEUROTROPHIC FACTOR RECEPTOR RET AND SECONDARY LYMPHOID ORGAN DEVELOPMENT

The development of enteric lymphoid structures, such as CP and PP depends on the transcription factors ID2, RORγt and AHR and it was also show that the formation of gastrointestinal lymphoid organs requires the coordinate development of tissues from different embryonic layers[44]. Glial-derived neurotrophic factors activate the tyrosine kinase receptor RET and were show to be central players in gut development.

Ret is widely expressed in the central and enteric nervous systems and is critical for the development and maintenance of several tissues and cell types, including the nervous system, kidney, adrenal glands, testicles, thyroid and some restricted subsets of haematopoietic cells[84].

RET was shown to be expressed in various lymphoid tissues and immune cells[85,86,87,88,89,90,91,92,93], but their functional significance has been only demonstrated in CD11c^{pos}, Lymphoid Tissue initiator cells in the embryo[94,95] and more recently in haematopoietic stem cells[96].

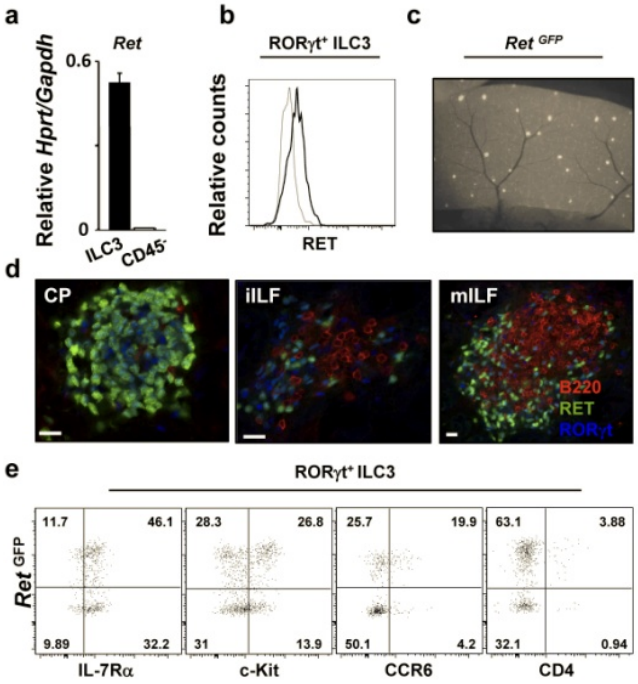

Figure 6 – Gut ILC3 express RET. a. Cells were FACS sorted and analysed by quantitative RT-PCR **b.** RET expression in RORγt^{pos} ILC3. **c-d.**. *Ret*^{GFP} intestines. Stereo (c.) and confocal (d.) microscopy. **e**. FACS analysis of intestinal lamina propia RORγt^{pos} ILC from *Ret*^{GFP} intestines.

The critical importance of RET is also highlighted by the high degree of protein conservation in several species, ranging from *Drosophila* to higher primates[84,97]. *Ret* encodes a transmembrane tyrosine kinase receptor protein that is composed of three domains: an extracellular ligand-binding domain, with four cadherin-like repeats and a cysteine-rich region; a hydrophobic transmembrane domain; and a cytoplasmic portion with a tyrosine kinase domain[84]. The glial-derived neurotrophic factor (GDNF) and three related GDNF-family ligands (GFL), i.e, neurturin (NRTN), artemin (ARTN)

and persephin (PSPN), signal through the RET tyrosine kinase receptor. The specificity of the RET/GFL axis is determined by one of the four GDNF family α co-receptors (GFRα): GFRα1 binds preferentially to GDNF, GFRα2 to NRTN, GFRα3 to ARTN and GFRα4 to PSPN[84]. GFL bind to GFRα, and the GFL-GFRα complex then interacts with two RET molecules, which form a homodimer and induce signal transduction through ERK1/2, PI3K/Akt and p38/MAP kinase activation[84]. GFL are produced as precursors that are cleaved and activated upon secretion[84,98]. They bind to proteoglycans of the extracellular matrix, which increase their local concentration and restrict diffusion[84,98].

Mouse models with homozygous null mutations of *Ret* are peri-natally lethal, and *Ret* null embryos exhibit a wide range of developmental abnormalities affecting the nervous system, kidney and Peyer's patches[94,95,99]. In humans, *Ret* mutations have been linked to various diseases. Activating mutations of *Ret* lead to cancer, i.e., somatic chromosomal rearrangements result in Papillary Thyroid Carcinoma and point mutations on the extra or intracellular domain of RET are responsible for Multiple Endocrine Neoplasia 2 syndrome (MEN2A and MEN2B)[84]. On the other hand, hypomorphic inactivating mutations of *Ret* lead to Hirschsprung's disease or congenital intestinal aganglionosis[98,100], which is characterised by defects in enteric innervations and megacolon[98,100].

Strikingly, while foetal ILC3 lack RET expression[95], our initial studies indicated that adult enteric ILC3 express this kinase (Figure 6).

5. AIMS OF THE THESIS

Enteric homeostasis requires a complex interplay between ILC and cells in their surroundings, but how ILC perceive, integrate and respond to local environmental cues remains poorly understood. ILC produce cytokines that are critical to tissue homeostasis, but are also essential for immune responses to intestinal microbes.

In this thesis, we hypothesized that ILC3-autonomous neuroregulatory signals control intestinal defence and glial-derived neurotrophic factors control ILC3 function, mucosal homeostasis and microbiota composition.

Initially, we manipulated RET signals in inflammation using *Ret* conditional loss (*Rorc*-Cre/*Ret*$^{fl/fl}$) and gain of function (*Ret*^{MEN2B}) mouse models. With this strategy, we assessed the role of RET-dependent neurotrophic factors in ILC and epithelial cell function in health and disease.

Deregulation of intestinal homeostasis and propensity to enteric inflammation has been associated with dysbiosis. Thus, we hypothesised that ILC3-specific neurotrophic factor signals may be critical regulators of intestinal microbial ecology. To this end, faeces and colonic biofilms from *Ret* conditional knock-out mice were isolated at steady-state and during inflammation and sequentially analysed by pyrosequencing.

With our work, we aimed at defining if neuroregulatory signals act as ILC3 environmental sensors, integrating commensal-derived signals into tissue protective innate functions that preserve normal host-microbial interactions and intestinal homeostasis.

V. MATERIALS AND METHODS

1. MOUSE STRAINS

RET loss of function was analysed using *Ret* conditional knock-out mice[96,101]. Our targeting strategy was based on the use of Cre-LoxP recombination. *Ret* floxP mice were bred to *Rorc*-Cre[44] transgenic mice to delete the floxed allele in RORγt^{pos} cells (Figure 7). RET gain of function was analysed using *Ret*^{MEN2B} mice, that have a constitutive sate of ligand dependent RET activation[102]. All mouse strains were bred and maintained at the IMM animal facility. All procedures and experiments were performed accordingly to institutional and national guidelines.

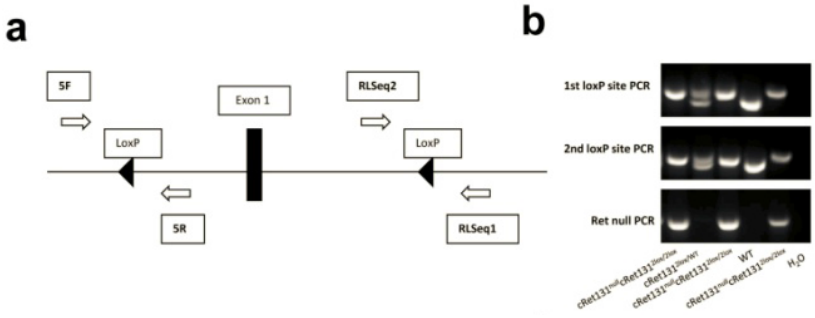

Figure 7 – Conditional models for *Ret*. **a.** *Ret conditional* knock-out construct **b.** *Ret*$^{fl/fl}$ mice DNA.

2. GENOTYPING POLIMERASE CHAIN REACTION (PCR)

DNA isolation was performed by briefly keeping of snipped tails in Tail Lysis Buffer (Annex I) and 0.4mg/mL of Proteinase K (Promega) at 56ºC until the tissue was completely digested. DNA was extracted by centrifuging the samples with Isopropanol (Sigma-Aldrich) for 25min 4ºC and 13000rpm, and by washing the pellet with 70% Ethanol followed by centrifugation at 13000rpm for 10min at 4ºC. DNA was ressuspended in Milli-Q water. DNA amplification was performed on a Veriti 96-Well Thermal Cycler (Applied Biosystems). Extracted DNA was amplified according to the following protocol: 25mM MgCl, 1.0mM dNTPs, 10x PCR Gold Buffer, Amplitaq® Gold DNA Polymerase (Applied Biosystems) and 0.25µM specific primers (Annex II) in a 20µL volume. The PCR program used consisted of an initial step of polymerase heat activation, performed at 95ºC during 10min, followed by 35 amplification cycles consisting of 3 distinct steps (DNA denaturation – 94º C for 30 sec, DNA-primer annealing step – 60º C for 45 sec, and polymerase reaction elongation – 72ºC for 1

min.), and a final step of DNA extension at 72º C for 10 min. Primers sequences are detailed in Annex II. After PCR reaction amplified products were resolved in a 1.5% agarose gel containing Gel Red (Biotium).

3. CELL ISOLATION, STAINING AND FLOW CYTOMETRY

For adult ILCs isolation, 8-weeks old mice were dissected and the intestines removed. After flushing the intestines content with PBS (Annex I), lamina propria cells were isolate as previously described[75]. For staining of surface markers, cells were initially incubated with Fc block in FACS buffer (Annex I), in order to prevent non-specific binding of antibodies (Ab) to Fc receptors. All staining steps were performed on ice and in the dark. For staining of nuclear factors, cells were fixed in Fixation Buffer (eBiosciences) for 20 min and permeabilised in 1X Perm Buffer (Annex I) for 5 min at room temperature. Cells were then incubated with anti-RORγt (AFKJS-9) for 30 minutes and washed in Perm Buffer. For cytokine staining, cells were previously incubated with PMA (Sigma-Aldrich)/Ionomycin and Brefeldin A (eBioscience) (Annex I) for 4h and 30min at 37º C, in order to activate cytokine production and block their secretion. Data analysis was performed using FlowJo software. Used Abs were: anti-NKp46 (29A1.4), anti-RORγt (AFKJS-9), (eBio17B7), anti-CD4 (RM4.5), anti-TCRβ (H57-597), anti-Thy1.2 (53-2.1), anti-CD45 (30-F11), anti-CD19 (MB19-1), anti-CD3 (eBio500A2), anti-Gr-1 (RB6-8C5), anti-CD11c (N418), anti-TER119 (TER-119), anti-IL-22 (1H8PWSR) and Rat IgG1 iso control (eBRG1) from eBiosciences; and anti-CD8b (4TS156.7.7), anti-CD8a (53-6.7) from Biolegend.

4. GENERATION OF MOUSE CHIMERAS

Rag$^{-/-}$*γc*$^{-/-}$ recipients were irradiated with two doses of 450rad with a minimum two hours interval. Mice were injected with 0.5x10^{6} lin^{neg} ckit^{pos} viable foetal liver cells from either E14.5 *Ret*$^{GFP/GFP}$ or *Ret*$^{WT/GFP}$ littermate controls. Mice were either analised 8 weeks post-transplantation.

5. HISTOPATHOLOGY

Animals were sacrificed in a CO_2 chamber and necropsy performed. The gastrointestinal tract was isolated and the colon separated from the caecum. Samples of colon and caecum were placed into 10% neutral buffered formalin. Transverse sections of the colon and longitudinal sections of the caecum were routine-processed for paraffin embedding and stained with Hematoxylin/Eosin and Gram (to visualize bacteria). Tissue sections were examined by a pathologist, blinded to experimental groups, in a Leica DM2500 microscope coupled to a Leica MC170 HD microscope camera.

6. DEXTRAN SODIUM SULFATE (DSS)-INDUCED COLITIS

Chemically induced colitis in mice was performed by administration of 2.5% (w/v) DSS (molecular mass = 36,000-50,000 Da; MP Biomedicals) in drinking water for 7 days following by 5 days of plain water preceding analysis.

7. *Citrobacter rodentium* INFECTION

Mice were infected with the enteric pathogen *Citrobacter rodentium*. Before infection, mice were treated with a ”cocktail” of antibiotics in drinking water containing ampicillin (Sigma-Aldrich) (1 g/l), colistin (Sigma-Aldrich) (1g/l) and streptomycin (Sigma-Aldrich) (5g/l) (Sigma-Aldrich), supplemented with 3% sucrose (Sigma-Aldrich) 2 weeks before infection[31]. Infection procedures were done by gavages inoculation with $2x10^9$ colony forming units of *C. rodentium ICC180* (derived from DBS100, reporter strain[103]). Throughout infection, weight loss, diarrhea or bloody stools were monitored daily. Mice were sacrificed 6 days after infection. Gut samples were analysed by confocal microscopy and enteric lamina propria cells were analysed by flow cytometry.

8. GENE EXPRESSION ANALYSIS

In order to analyse the expression of mucins, beta-defensins and antimicrobial peptides, we isolated enteric epithelial cells using microscope assisted dissection[95]. Expression of the relevant genes was determined by quantitative RT-PCR as previously described[45,96]. In brief, RNA was extracted from cell suspension using RNeasy Mini Kit and RNeasy Micro Kit (Qiagen) and was retro-transcribed using High Capacity RNA-to cDNA Kit (Applied Biosystems), followed by a pre-amplification PCR using TaqMan PreAmp Master Mix (Applied Biosystems). TaqMan Gene Expression Master Mix was used in real-time PCR. TaqMan Gene Expression Assays for the relevant genes was obtained from Applied Biosystems. Gene expression levels were normalised to *Gapdh* and *Hprt1* (Annex III) and the follow genes were analysed: *Reg3a*, *Reg3b*, *Reg3g*, *Defa1*, *Defars1*, *Defa5*, *Defa21*, *Muc1*, *Muc3* and *Muc13* (Annex III).

9. SAMPLE COLLECTION AND PROCESSING

Stool samples were collected from mice and were stored at 4°C (limited storage time was encouraged) or processed immediately. Organ samples (mesenteric lymph nodes, liver and spleen) were sterilely removed at necropsy. All samples were mechanically homogenised in sterile PBS (Annex I).

10. COLONY FORMING UNITS MEASUREMENT

Organs were harvested, weighted, and brought into suspension. Bacterial colony forming units (CFU) were determined per volume, gram of tissue and total organ. CFU of faeces and organs samples were determined via serial dilutions on Luria Broth (LB) agar (Invitrogen). Colonies were counted after 2 days of culture at 37ºC. Bacterial translocation was determined in mesenteric lymph nodes, liver and spleen.
For *C. rodentium* infection, bacterial translocation was determined in mesenteric lymph nodes, liver and spleen taking in account total bacteria and Luciferace^{pos} *C.*

rodentium. CFU of faeces and organs samples of *C. rodentium* infected mice, were determined via serial dilutions in Luria Broth (LB) Agar (Invitrogen) and MacConkey Agar (Sigma-Aldrich). Processed samples were stored at -80°C until further analysis.

11. 454 SEQUENCING

Faeces were isolated from adult mice. Microbial DNA was isolated and bacterial 16S rRNA amplified by PCR using universal and bar-coded primers around the V5 and V6 hypervariable regions. Pyrosequencing was done using 454 Roche technology. Sequence analysis was performed using the QIIME platform112. Taxonomic categories were defined using RDP. Community structure analysis was done using UniFrac113 and principal coordinate analysis. Microbiome analysis was performed with the support from Dr. Elizabeth Grice, University of Pennsylvania, who is an expert in metagenomic analysis.

12. STATISTICS

Statistical analysis was performed using two-tailed *F*-test analysis of variance and two tailed Student's *t*-test. A *p*-value of <0.05 was considered significant. Results were scored as: * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

VI. RESULTS

1. RET DRIVES INNATE IL-22 PRODUCTION IN THE GUT MUCOSA

Studies in murine models identified a critical role for the cytokine IL-22 in regulating intestinal immunity, inflammation, and tissue repair[104]. In order to investigate whether RET controlled ILC3 function, we transplanted *Ret*$^{WT/GFP}$ or *Ret*$^{GFP/GFP}$ foetal liver cells into ILC deficient *Rag*$^{-/-}\gamma c^{-/-}$ hosts. We found that IL-22 expressing ILC3 were largely reduced in *Ret*$^{GFP/GFP}$ chimeras despite normal IL-22 production from T cells (Figure 8a-b,e). In agreement, analysis of *Ret*^{MEN2B} mice, with constitutively activated RET[102], in steady-state (Figure 8c-d) and upon DSS-induced colitis (Figure 10c-d), revealed a selective increase of IL-22^{pos} ILC3s while their IL17^{pos} counterparts were unaffected (Figure 8c-d).

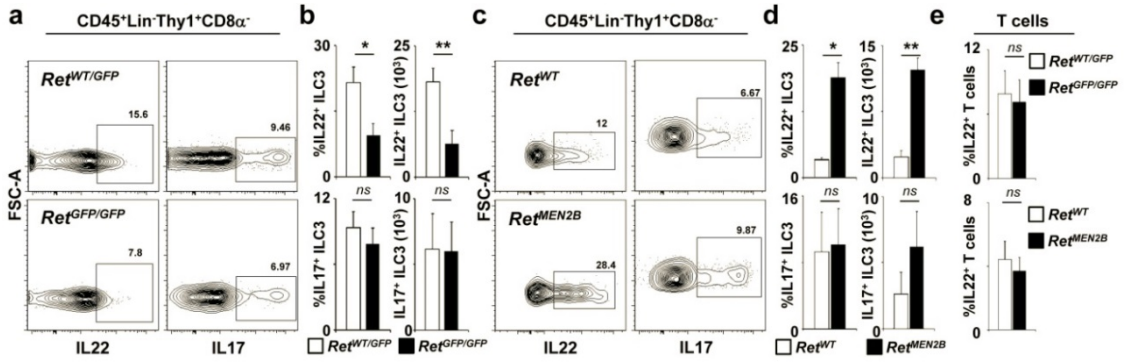

Figure 8 – RET drives innate IL-22 production. a-b. CD45.2 *Ret*$^{WT/GFP}$ or *Ret*$^{GFP/GFP}$ foetal liver cells were transplanted into *Rag*$^{-/-}\gamma c^{-/-}$ and analysed at 8 weeks. **a.** FACS analysis. **b.** Percentage and cell number/intestine of IL-22^{pos} and IL-17^{pos} ILC3 (n=12). **c.** FACS analysis of *Ret*^{WT} or *Ret*^{MEN2B} mice. **d.** Percentage and cell number/intestine of IL-22^{pos} and IL-17^{pos} ILC3 (n=10). **e.** Percentage of IL-22^{pos} T cells in *Ret*$^{WT/GFP}$ and *Ret*$^{GFP/GFP}$ chimeras (n=12.), and *Ret*^{WT} and *Ret*^{MEN2B} mice (n=10). *P<0.05; **P<0.01; ns not significant.

To further characterise the role of *Ret* in RORγt^{pos} ILCs, we generated a mouse line with a RORγt^{pos} ILCs cell-autonomous deletion of *Ret*. To achieve that we have crossed (mice with floxed *Ret* (*Ret*$^{fl/fl}$) with mice expressing Cre recombinase under the control of the *Rorc* promoter (*Rorc*-Cre)[105]. We obtained conditional knockout (KO) mice (*Rorc*-Cre/*Ret*$^{fl/fl}$), where *Ret* is deleted only in RORγt^{pos} ILCs and T cells.

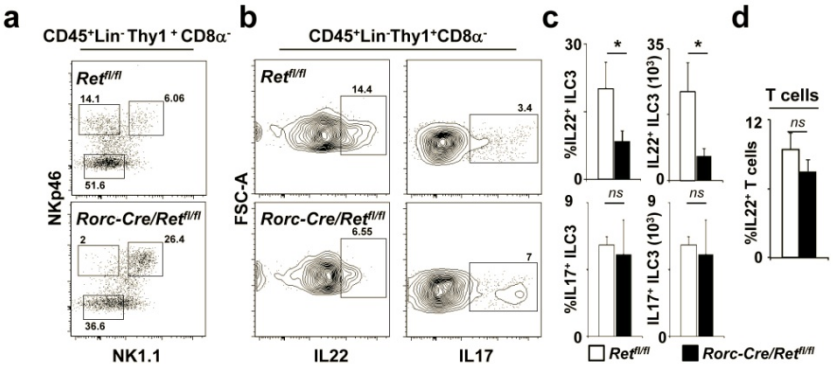

Figure 9 – ILC3-autonomous RET signals control IL-22. *Ret*$^{fl/fl}$ or *Rorc*-Cre/*Ret*$^{fl/fl}$ mice were studied. **a.** FACS analysis for NKp46^{pos} ILC3. **b.** Cytokine analysis. **c.** IL-22^{pos} and IL-17^{pos} ILC3. **d.** Percentage of IL-22^{pos} T cells. *P<0.05; ns not significant.

Analysis of these mice in steady-state (Figure 9c-d) and upon DSS-induced colitis (Figure 10a,b) showed a reduction of NCR^{pos} ILC3 when compared to WT littermate controls (*Ret*$^{fl/fl}$) (Figure 9a). In agreement, *Rorc*-Cre/*Ret*$^{fl/fl}$ ILC3 had reduced expression of IL-22, while their IL17^{pos} counterparts were unaffected (Figure 9b-c). Noteworthy, and *Rorc*-Cre/*Ret*$^{fl/fl}$ T cells had normal IL-22 levels (Figure 9d,8e).

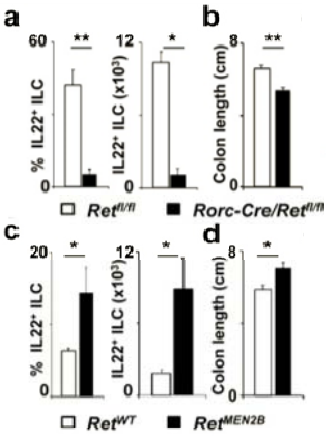

Figure 10 – DSS-induced colitis. a-c. *Rorc*-Cre/*Ret*$^{fl/fl}$ mice were DSS treated (n=4). **a.** percentage and number of IL-22^{pos} ILC3. **b.** Colon length. **d-f.** *Ret*^{MEN2B} mice were DSS treated (n=4). **c.** Percentage and number of IL-22^{pos} ILC3. **d.** Colon length. *P<0.05; **P<0.01.

2. RET DEFICIENT ILC3 SHAPE EPITHELIAL CELL HOMEOSTASIS

Previous reports have shown that IL-22 production has a protective effect in the gut through the production of antimicrobial peptides (β-defensin, RegIIIγ, RegIIIβ) by epithelia cells[7,77,104]. Thus, we investigated whether RET deficiency in RORγt expressing cells modulates reactivity genes in epithelial cells. Analysis of these gene transcripts in the epithelium of *Rorc*-Cre/*Ret*$^{fl/fl}$ mice, in steady-state, reveals a severe reduction of their expression when compared to their WT littermate controls (Figure 11a). This striking phenotype was further enhanced by the induction of inflammation after DSS treatment (Figure 11b). Notably, RT-PCR analysis showed a pronounced decrease in *Muc1*, *Muc3*, *Muc10*, and *Muc13*, which are involved in mucus layer formation.

Interestingly, analysis of gut epithelial cells from mice with a gain-of-function mutation of RET (*Ret*^{MEN2B}), where a single point mutation (Met919Thr) was introduced into the endogenous *Ret* gene locus, revealed consistent upregulation of epithelial reactivity genes (Figure 11a-b).

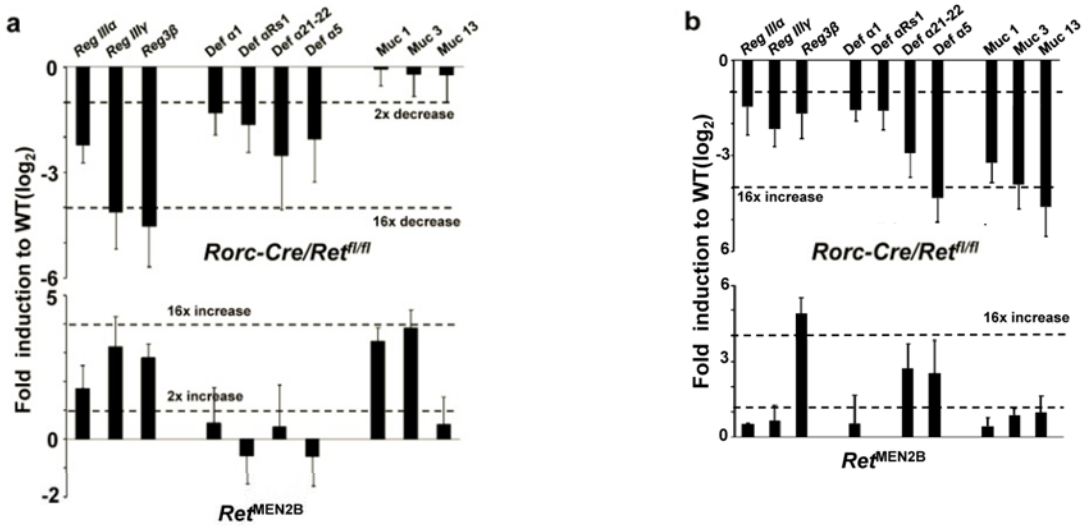

Figure 11 – a.ILC3 cell-autonomous RET signals control epithelial cell reactivity. 8 week old *Ret*$^{fl/fl}$ or *Rorc*-Cre/*Ret*$^{fl/fl}$ mice were studied. Quantitative RT-PCR analysis of enteric epithelial cells. Results show:Log_2 fold induction of: Top, *Rorc*-Cre/*Ret*$^{fl/fl}$ to *Ret*$^{fl/fl}$ cells; Bottom, *Ret*^{MEN2B} to *Ret*^{WT}.**b. ILC3 RET signals control epithelial cell reactivity during DSS-induced colitis.** 8 week old *Ret*$^{fl/fl}$ or *Rorc*-Cre/*Ret*$^{fl/fl}$ mice were DSS treated. Quantitative RT-PCR analysis of enteric epithelial cells. Results show:Log_2 fold induction of: Top, *Rorc*-Cre/*Ret*$^{fl/fl}$ to *Ret*$^{fl/fl}$ cells; Bottom, *Ret*^{MEN2B} to *Ret*^{WT}.

3. RET SIGNALS ARE CRITICAL TO CONTROL ENTERIC INFLAMMATION

Anatomical containment is essential to limit inflammation and to maintain normal systemic immune cell homeostasis. ILCs are distributed in the intestinal lamina propria and GALT, in which they are poised to respond rapidly to microbes that penetrate the epithelial barrier[5]. In addition, ILC3 were shown to promote epithelial integrity and homeostasis[7]. Failure to contain enteric bacterial communities lead to dissemination of commensal bacteria to peripheral organs, which in turn may promote multiple chronic infectious and inflammatory diseases, including progressive ulcerative colitis and inflammatory bowel disease[104].

Our initial observations that RET signals control innate IL-22 expression and epithelial cell reactivity lead us to interrogate whether RET^{pos} ILC3 also set the inflammatory responses in the gut wall. Thus, *Ret*$^{fl/fl}$ and *Rorc*-Cre/*Ret*$^{fl/fl}$ mice were treated with DSS and their intestines were sequential analysed by microscopy. Histopathology analysis indicated that both *Rorc*-Cre/*Ret*$^{fl/fl}$ mice developed more severe inflammation than their WT littermate controls (Figure 12). Inflammation in *Rorc*-Cre/*Ret*$^{fl/fl}$ mice was characterised by multifocal aggregates of inflammatory cells that were found in the mucosa and extending to the submucosa (Figure 12f). *Rorc*-Cre/*Ret*$^{fl/fl}$ mice also displayed significant areas of ulceration (Figure 12e-g).

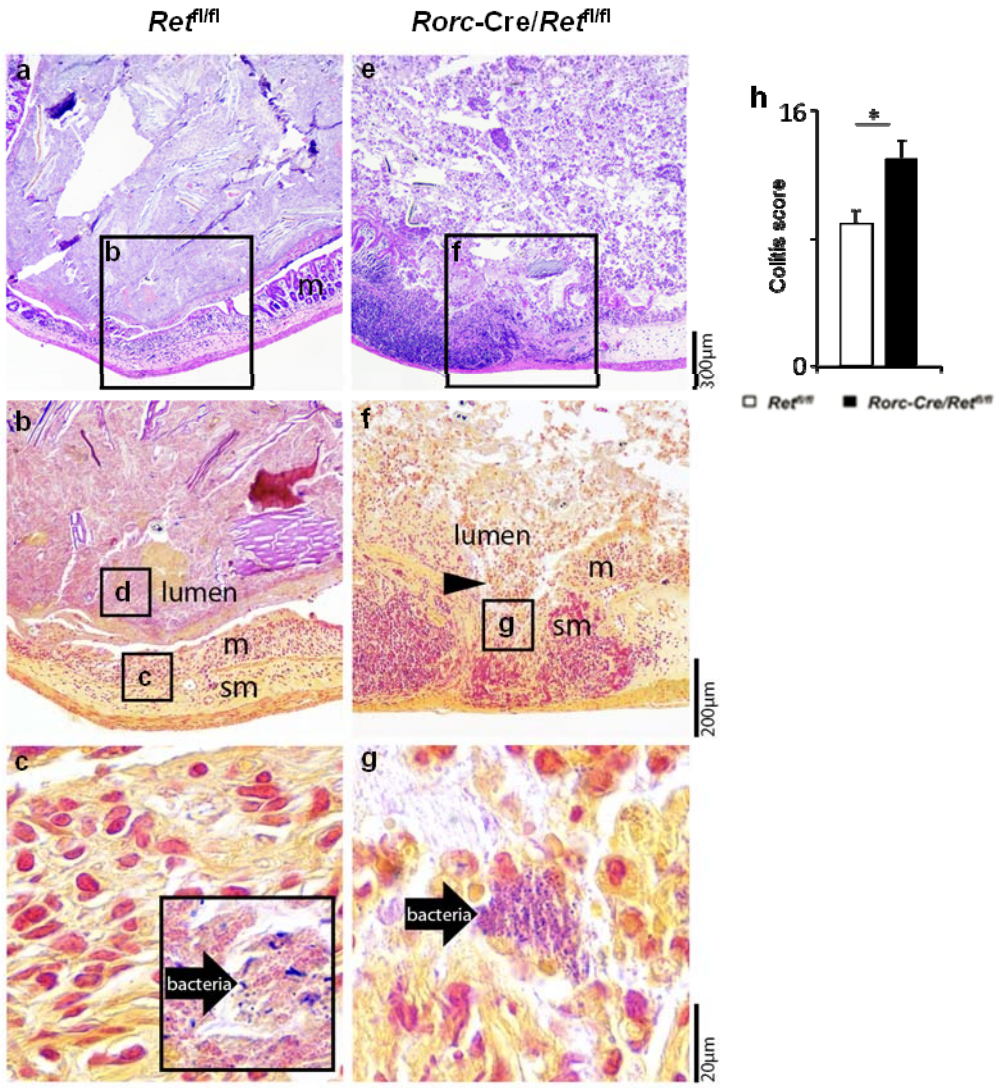

Figure 12 – Histopathology of acute colitis induced by DSS in *Ret*$^{fl/fl}$ and *Rorc*-Cre/*Ret*$^{fl/fl}$ mice. a. The caecum of *Ret*$^{fl/fl}$ mice shows mild and focal erosive mucosal loss and effacement by inflammatory cells, adjacent to intact mucosa (m). **b-d**. Coccoid and filamentous bacteria are only seen in the lumen of the caecum (arrow), with no infiltration of the mucosa. **e**. The caecum of *Rorc*-Cre/*Ret*$^{fl/fl}$ mice shows more severe lesions, with ulcer formation (inset); and the mucosa adjacent to the ulcer shows irregular glands. **f**. Exudate adherent to ulcerated mucosa contains degenerate inflammatory cells, fibrin and coccoid bacterial colonies (arrowhead), with a portion escaping into the adjacent lamina propria/submucosa (sm) (arrow) (**g**). **h.** Colitis score[106]. *a, e, Hematoxylin and Eosin staining; b, c, d, f and g, Gram staining.* *P<0.05.

Analysis of RET gain-of function mutants (*Ret*^{MEN2B}) revealed that these mice were highly protected over their WT littermate controls. Assessment of colonic histology revealed markedly decreased inflammation when compared to their WT littermate controls (Figure 13a,d). Overall, these data indicate that activation of RET expressing RORγt cells by neurotrophic factors is essential to control enteric inflammation.

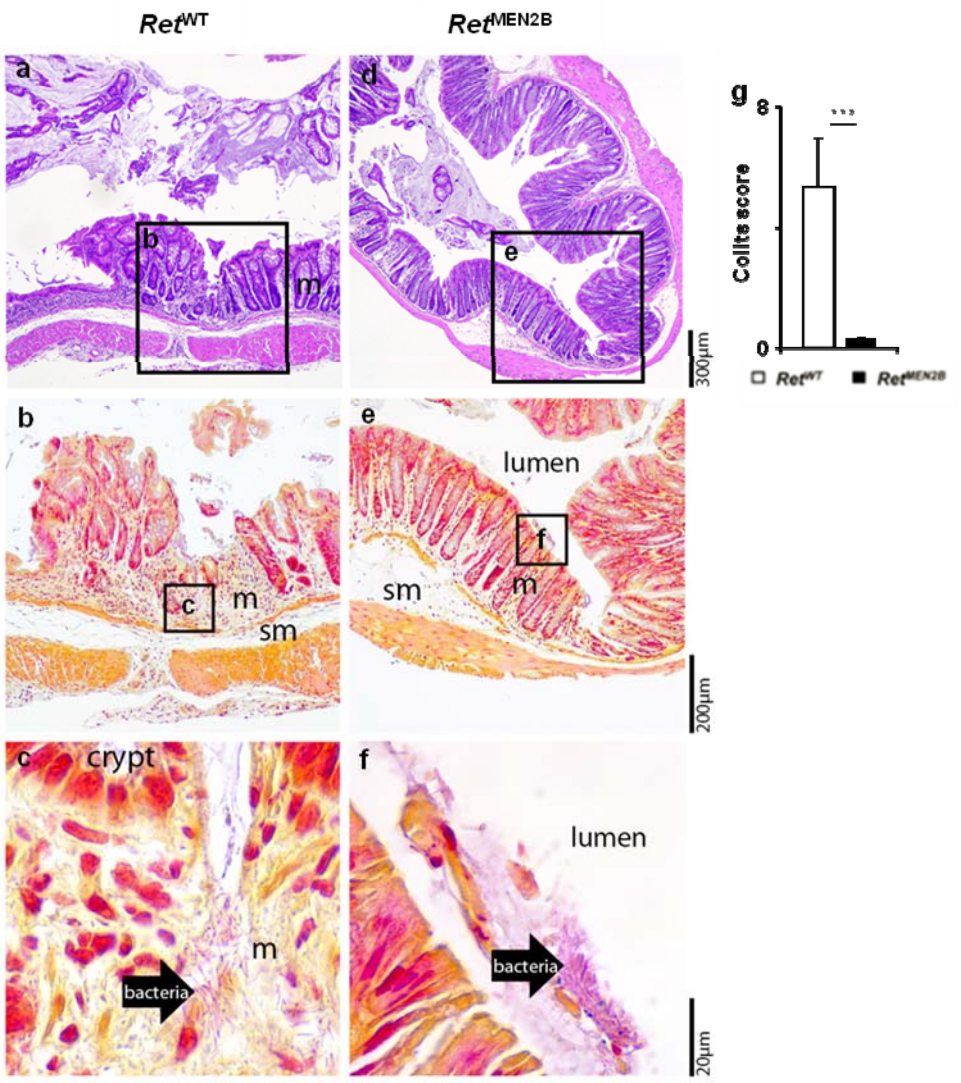

Figure 13 – Histopathology of acute typhlocolitis induced by DSS in *Ret*^{WT} and *Ret*^{MEN2B} mice. **a**. The colon of *Ret*^{WT} mice shows mild and focal erosive mucosal loss and effacement by inflammatory cells, adjacent to intact mucosa (m). **b-c**. Filamentous bacteria are seen in the lumen of the colon, associated with focal infiltration of the mucosa (**c**). **d-f**. The colon of *Ret*^{MEN2B} mice shows minimal lesions, and bacterial colonies are restricted to the lumen (**f**). **g.** Colitis score[106]. *a, e, Hematoxylin and Eosin staining; b, c, d, f and g, Gram staining. m, mucosa; sm, submucosa.* ;***P<0.001.

4. RET SIGNALS ARE CRITICAL TO CONTROL *Citrobacter rodentium* INFECTION

Since DSS does not directly mimic common inflammatory/infectious pathologies, we also tested whether RET signals were required for the control of attaching and effacing intestinal infections, such as *Citrobacter rodentium*[64]. *Citrobacter rodentium* is a natural pathogen of mice that is used to model human infections with Enteropathogenic (EPEC) and Enterohemorragic (EHEC) bacteria [16].

Ret$^{fl/fl}$ and *Rorc*-Cre/*Ret*$^{fl/fl}$ mice were orally inoculated with *C. rodentium* (Figure 14). At 24h after infection both groups had similar colonization by *C. rodentium*, however these levels increased progressively and *Rorc*-Cre/*Ret*$^{fl/fl}$ mice had significantly increased *C. rodentium* 72h after infection when compared to their WT counterparts (Figure 14). Thus, we concluded that activation of RET expressing RORγt cells by neurotrophic factors is essential to control enteric infection by attaching and effacing bacteria.

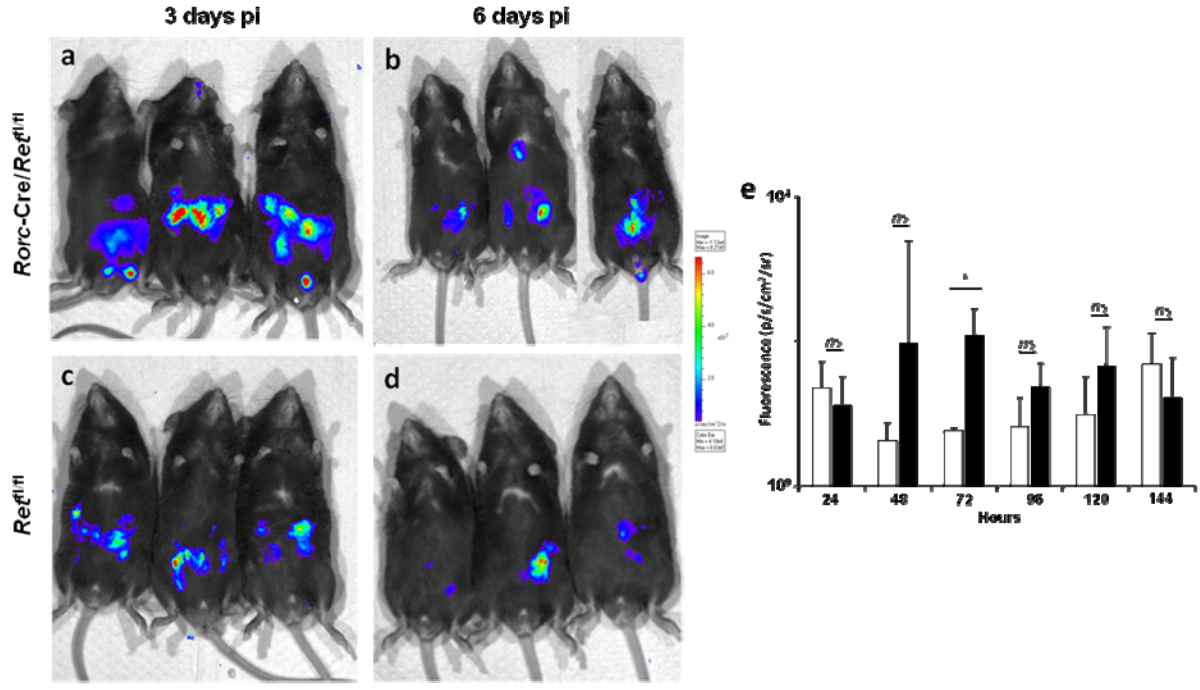

Figure 14 – Monitoring of transmission of the gastrointestinal pathogen *Citrobacter rodentium* through the oral gavages. Mice were exposed to *C. rodentium ICC180* (derived from DBS100). Whole-body imaging of *Rorc*-Cre/*Ret*$^{fl/fl}$ mice, 3 days **(a)** and 6 days **(b)** post-infection with 2 x 10^9 CFU of bioluminescent. Whole-body imaging of *Ret*$^{fl/fl}$ mice, 3 days **(c)** and 6 days **(d)** post-infection with 2 x 10^9 CFU of bioluminescent. **e.** *Citrobacter rodentium* fluorescence. The same three mice were imaged in each 24h after infection. Images were acquired using an IVIS system (Caliper Life Sciences) with an integration time of 1 min and are displayed as pseudocolour images of peak bioluminescence, with variations in colour representing light intensity at a given location. Red represents the most intense light emission, while blue corresponds to the weakest signal. The colour bar indicates relative signal intensity (as photons s^{-1} cm^{-2} sr^{-1}). *P<0.05; ns not significant.

5. RET SIGNALS ARE CRITICAL TO THE ANATOMICAL CONTAINMENT OF COMMENSAL BACTERIA

Anatomical containment of commensal bacteria is crucial to limit inflammation. Our observations that RET signals control innate IL-22 expression, epithelial cell reactivity and gut inflammation lead us to interrogate whether RET signals were also required for the anatomical containment of commensal bacteria. Thus, *Ret*$^{fl/fl}$ and *Rorc*-Cre/*Ret*$^{fl/fl}$ mice were treated with DSS and their peripheral organs, notably mesenteric lymph nodes, liver and spleen, were analysed for the presence of translocated bacteria (Figure 15a-b).

Strikingly, while DSS treated *Rorc*-Cre/*Ret*$^{fl/fl}$ mice had increased bacterial translocation from the gastro-intestinal tract to peripheral organs (lymph nodes, liver and spleen) (Figure 15a), RET gain-of function mutants (*Ret*^{MEN2B}) were highly protected over their WT littermate controls and showed limited bacterial translocation (Figure 15b).

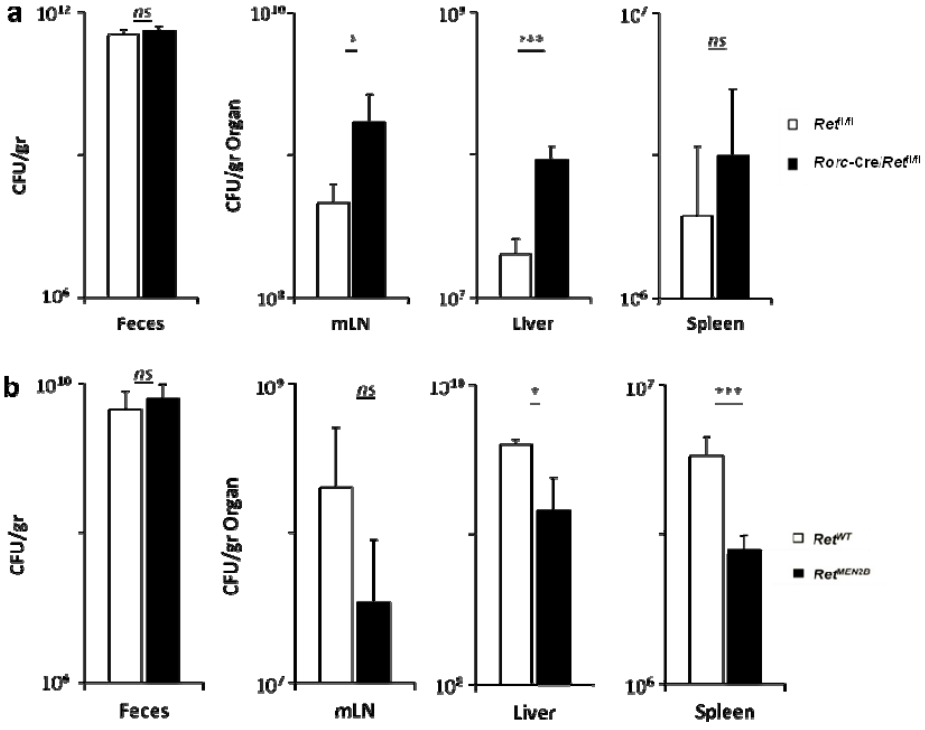

Figure 15 – DSS colitis promote translocation of bacteria to peripheral organs. a. Bacterial load (colony-forming unit) present in faeces, mesenteric lymph nodes (mLN), liver and spleen of DSS treated in *Rorc*-Cre/*Ret*$^{fl/fl}$ and *Ret*$^{fl/fl}$ mice. **b.** Bacterial load (colony-forming unit) present in faeces, mesenteric lymph nodes (mLN), liver and spleen of DSS treated in *Ret*^{MEN2B} and *Ret*^{WT} mice. *P<0.05; **P<0.01;***P<0.001, ns not significant.

6. RET DEFICIENT MICE DISPLAY ALTERED COMMENSAL MICROBIOTA

The aberrant IL-22 production and epithelial defence of *Rorc*-Cre/*Ret*$^{fl/fl}$ mice led us to investigate the composition of symbiotic microbes. Thus, we used bacterial 16S rRNA gene pyrosequencing from DNA material collected from focal samples of *Ret*$^{fl/fl}$ and *Rorc*-Cre/*Ret*$^{fl/fl}$ mice, before, during and after DSS treatment (Figure 16a-d).

We obtained 69,788 high-quality sequences with an average ~2326 reads/sample (Figure 16e-f). 159,554 operation taxonomic units (OTUs) and Shannon-Wiener Diversity Index were calculated for each sample (Figure 17). Multivariable analysis of the colonic microbiome was performed by principal component analysis (PCA).

The OTU distribution was examined to rough calculation shared species between the two groups of animals in the three different time points. Results show that during DSS-induced inflammation *Rorc*-Cre/*Ret*$^{fl/fl}$ mice have a significant reduction in the number of commensal species, when compared to their WT littermate controls (Figure 16).

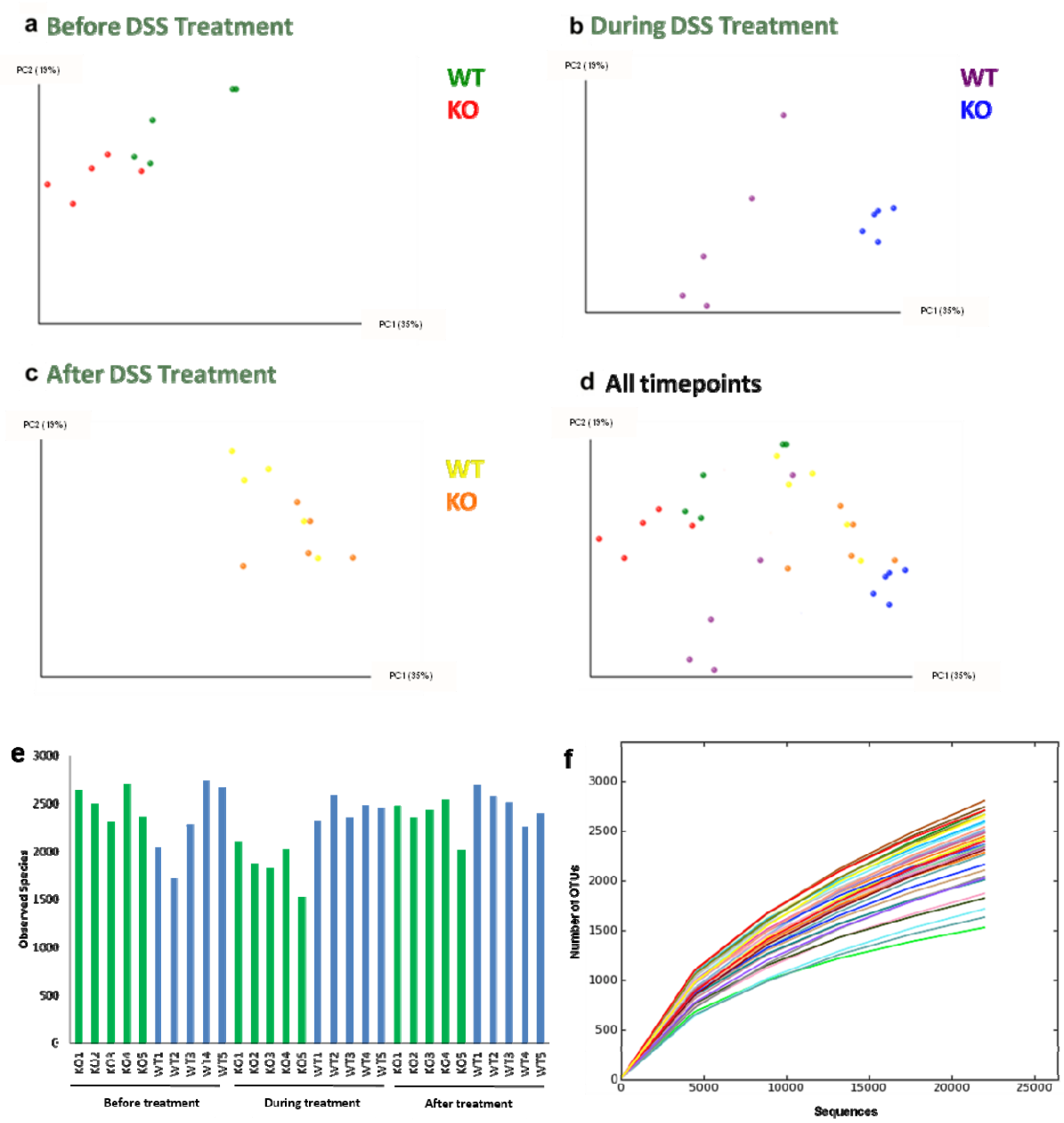

Figure 16 – *Ret* deficient mice have altered microbiota. Faecal samples were obtained and analysed by 16S rRNA. DNA was prepared and subjected to pyrosequencing. A Total of 69,788 sequences (observed species) was obtained from the 30 samples (n= 10/time point). **a.** Score plot of PCA of the microbiomes of *Ret*$^{fl/fl}$ and *Rorc*-Cre/*Ret*$^{fl/fl}$ mice based on the first three principal components in three different time points: **a**. before DSS; **b.** during DSS; **c.** after DSS; **d.** All time points. Each point represents one mouse. **e.** Taxon-based analysis at species level among the groups. Each bar represents one mouse in three different time points. Green bars represent conditional knock-out (*Rorc*-Cre/*Ret*$^{fl/fl}$) mice and blue bars represent control (*Ret*$^{fl/fl}$) mice. **f.** OTU rarefaction curves. Each line represents one mouse.

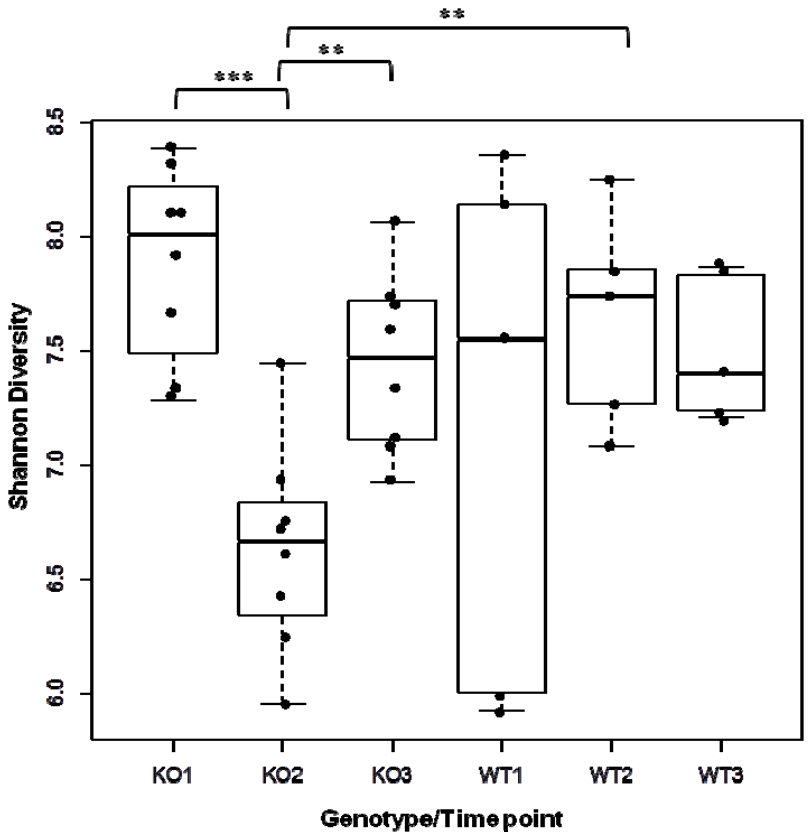

Figure 17 – Shannon Diversity. Difference among the two groups in three different time points. WT (*Ret*$^{fl/fl}$) and KO (*Rorc*-Cre/*Ret*$^{fl/fl}$). Time points: **1**- before DSS; **2**- during DSS; **3**- after DSS. **p <0.005, ***p<0.001.

Sequentially we analysed quantitative changes in the microbial communities from faecal samples by barcoded pyrosequencing of 16S rRNA. Taxon-based analysis revealed that the microbiome of both *Ret*$^{fl/fl}$ and *Rorc*-Cre/*Ret*$^{fl/fl}$ mice was mainly composed by Bacteriodetes, Firmicutes, Proteobacteria and Actinobacteria (Figure 16).

However, examination of the relative abundance of taxa in steady state revealed altered microbiota, notably increased levels of Proteobacteria (Figure18a-b). In contrast, during DSS-treatment, Firmicutes were strikingly reduced while Bacteroidetes were strongly increased in *Rorc*-Cre/*Ret*$^{fl/fl}$ mice when compared to their WT littermate controls (Figure 18c-d). Interestingly, after resolution of the DSS-induced enteric inflammation *Rorc*-Cre/*Ret*$^{fl/fl}$ mice the levels of each phylum became nearly similar in WT and *Rorc*-Cre/*Ret*$^{fl/fl}$ mice.

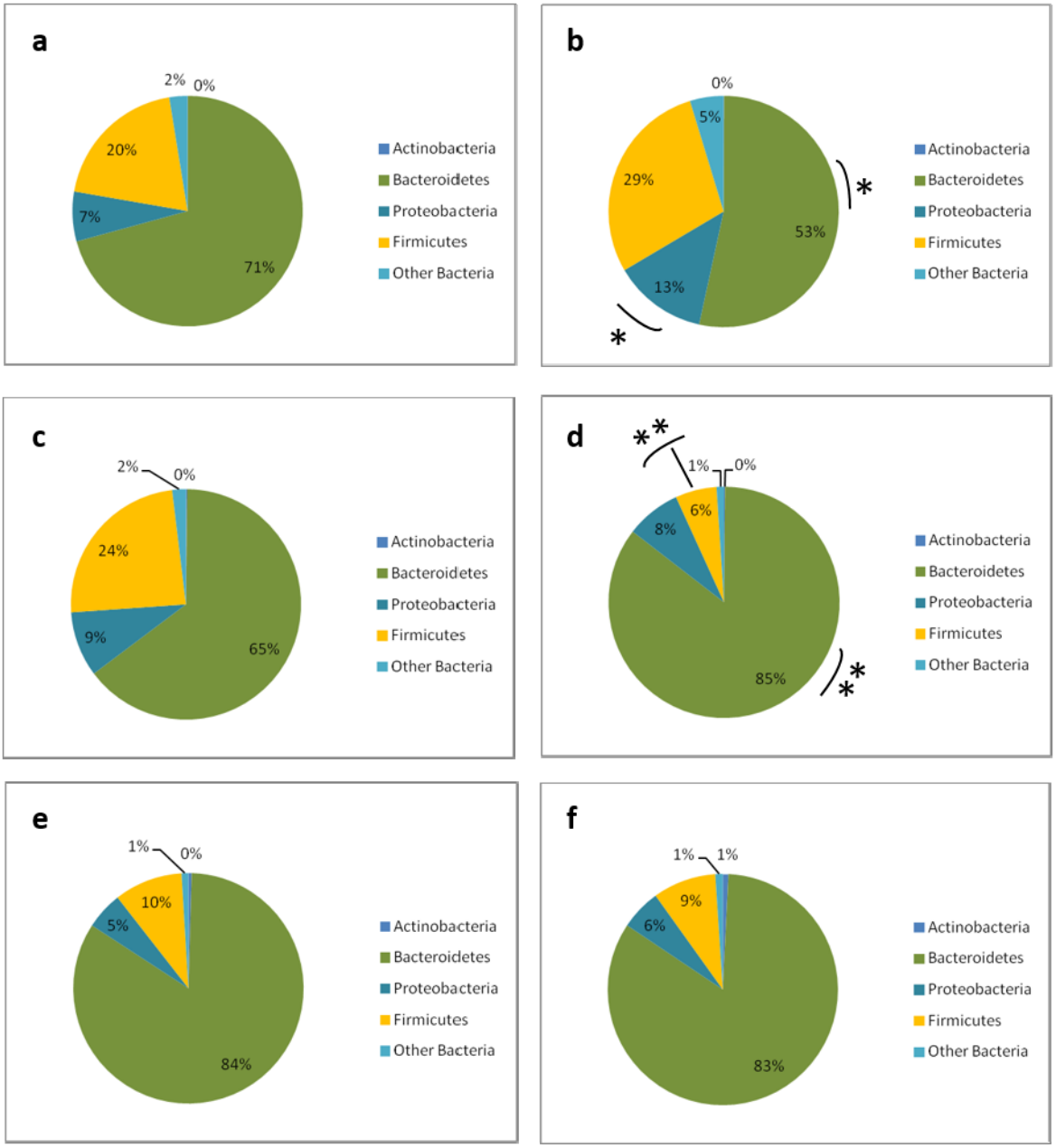

Figure 18 – ILC3 cell-autonomous RET signals control diversity of symbiotic microbiota during colitis. 5 animals of each group, *Ret*$^{fl/fl}$ and *Rorc*-Cre/*Ret*$^{fl/fl}$, with 8 weeks old WT mice are studied. Taxonomic distribution of the most abundant phyla in faeces collected from *Ret*$^{fl/fl}$ mice: **a.** before DSS treatment; **c.** during DSS treatment and **e.** after DSS treatment. Taxonomic distribution of the most abundant phyla in faeces collected from *Rorc*-Cre/*Ret*$^{fl/fl}$ mice: **b.** before DSS treatment; **d.** during DSS treatment and **f.** after DSS treatment. *P<0.05; **P<0.01;***P<0.001.

VII. DISCUSSION

An increasing number of studies have been unravelling the interactions between eukaryote organisms and their bacterial symbionts. Collectively, these studies showed that the intestinal microbiota can influence metabolic and immune pathways of the host, shaping human health and disease[107].

The intestinal epithelial barrier is a key element in the first-line of defence against luminal microbes and IL-22 expressing ILC3 were shown to be important for the maintenance of the intestinal epithelial cell barrier[81]. ILC3 development and function have been widely perceived to be programmed[20]. However, recent evidence indicates that ILC3 are also controlled by dietary signals[45,108]. Nevertheless, how ILC3 perceive, integrate and respond to environmental cues remains utterly unexplored. In addition, the relationship between ILC3 and the gut microbiota composition remain elusive.

In this work we interrogated whether neurotrophic factors, highly abundant molecules in the gut wall environment, modulate ILC3 function and microbiota composition. Altogether, our data indicate that the neurotrophic factor receptor RET is critical to innate IL-22 function, shaping gut homeostasis and microbial communities.

IL-22 is a central gut homeostasis cytokine and is one of the main ILC3 effector functions[21,109]. IL-22 acts on gut epithelial cells inducing the production of several antimicrobial peptides, including RegIIIγ[21]. Interestingly, this antimicrobial peptides (AMP) has been implicated in early immune responses to attaching and effacing bacteria, notably *Citrobacter rodentium* through binding to the peptidoglycan surfaces of Gram-positive bacteria[42].

Our data revealed that innate IL-22 expression is controlled by RET signals. In turn altered IL-22 expressing ILC3 correlated with a notable regulation of epithelial cell reactivity genes, known to be modulated by IL-22. Nevertheless, and despite normal IL-22 expression by T cells from *Rorc*-Cre/*Ret*$^{fl/fl}$ and *Ret*^{MEN2B} mice, it remains to be formally demonstrated whether abnormal production of ILC3 derived IL-22 in these models is the cause of altered expression of epithelial reactivity genes. In order to

test this hypothesis future studies could test whether complementation of RET deficient ILC3 with ectopic retroviral IL-22 restores epithelial reactivity genes.

In this work, we further revealed that in mice with DSS-induced colitis or infected with the attaching and effacing bacteria *C. rodentium*, RET signals are critical keepers of gut defence. While DSS treated *Rorc*-Cre/*Ret*$^{fl/fl}$ mice had reduced IL-22^{pos} ILC3, increased inflammation and bacterial translocation from the gastro-intestinal tract, RET gain-of function mutants (*Ret*^{MEN2B}) were highly protected over their WT littermate controls. Thus, it is likely that the low frequencies of IL-22 producing ILC3 in *Rorc*-Cre/*Ret*$^{fl/fl}$ mice may disrupt epithelial homeostasis, promoting intra- and extra-intestinal overgrowth and/or propagation of certain bacterial species to other tissues. Whether RET dependent innate IL-22 expression shapes the localization of commensal bacteria within the intestinal lumen remain elusive. However, the reduced levels of IL-22 and the complementary decrease in antimicrobial peptides and mucins, may allow commensal bacteria to be closer to the epithelium, where they are normally absent. Interestingly, in *Rorc*-Cre/*Ret*$^{fl/fl}$ mice DSS treatment disrupted the colonic epithelium, allowing significant numbers of commensal bacteria to come into contact with the mucosa and possibly to translocate to other organs (Figure 15a-b).

Analysis of the *Rorc*-Cre/*Ret*$^{fl/fl}$ microbiota revealed significant chances of both abundance relationships and diversity. In steady state condition, we observed that *Rorc*-Cre/*Ret*$^{fl/fl}$ mice had increased Proteobacteria, which have been previously shown to be increased in inflammatory disease patients[108]. Interestingly, upon DSS-induced colitis the phylum Firmicutes was preferentially reduced in RET conditional knock-out mice and the microbiome of these mice also had a decreased diversity. The phylum Firmicutes includes the family Lactobacillaceae, a Gram-positive family of lactic acid producing bacteria that is assumed to be normally present in a healthy flora (Figure 18b-c)[10,110]. Thus, it is possible that a reduced population of this beneficial microbiota may further facilitate the enteric inflammation of *Rorc*-Cre/*Ret*$^{fl/fl}$ mice.

Interestingly, *Rorc*-Cre/*Ret*$^{fl/fl}$ mice revealed the emergence of low levels of bacterial phylum that are usually absent or at extremely low levels in wild-type mice. These included TM7 (Figure 18). TM7 is an unculturable phylum that was identified by 16S

ribosomal RNA sequencing in environmental soil samples and has been since found in mouse faeces [111] and human oral cavity[24]. Proteobacteria are Gram-negative bacteria and contain important pathobiont genera such as *Escherichia*, *Salmonella*, *Vibrio* and *Helicobacter*[112]. Altogether, these findings suggest that activation of ILC3 by neurotrophic factors control innate IL-22 which in turn alter epithelial reactivity that may therefore induce dysbiosis. Future studies will address whether the microbiota of *Rorc*-Cre/*Ret*$^{fl/fl}$ mice is colitogenic and transmissible, thus shedding light on whether dysbiosis is a cause of altered innate IL-22.

In conclusion, our data indicate that ILC3-autonomous RET signals regulate intestinal defence. Thus, therapeutic targeting of RET expressing ILC3 can be potentially explored to promote tissue defence in infectious and inflammatory diseases that are major Public Health concerns.

VIII.ANNEXES

I.BUFFERS AND SOLUTIONS

Tail Lysis Buffer: 10mM Tris pH 8, 100mM NaCl, 10mM EDTA pH 8, 0.5% SDS in MilliQ water.

PBS 1X: 10% Phosphate Buffer Saline (PBS) 10X (GIBCO) in MilliQ water.

FACS Buffer: PBS 1X with 2% FBS (Foetal Buffer Saline).

Perm Buffer 1X: 10% Perm Buffer 10X (eBiosciences) in MilliQ water.

PMA/Ionomycin and Brefeldin A: phorbol 12-myristate 13-acetate/Ionomycin and Brefeldin A

4% PFA: 4g of Paraformaldehyde (PFA) (Sigma-Aldrich) in 100 mL of blocking Buffer

II.PRIMER SEQUENCES

Genotyping primers:

Rorc-cre Allele

Rorc-cre F: 5'– TTC CCG CAG AAC CTG AAG ATG TTC G – 3'
Rorc-cre R: 5'– GCC AGA TTA CGT ATA TCC TGG CAG C – 3'

c*Ret*131$^{flox/flox}$

5F: 5'– AAG CTC CCT CCT ACC GTG CT – 3'
5R: 5'– TGG GAT GAA CTC TGC CCA TT – 3'
RLseq1: 5'– TAC ATG CTG TCT GCT CTC AG – 3'
RLseq2: 5'– TGC TGC TCC ATA CAG ACA CA – 3'

Ret^{MEN2B} Allele

MEN2B F: 5'– GCT CAG TCT GAG ATG CTG GG – 3'
MEN2B R: 5'– CCT CTC ACA CAC CAC AAC C – 3'

III.PCR PROBES

qPCR probes:

Gene	Invitrogen Reference
Gapdh	Mm99999915_g1
Hprt	Mm00446968_m1
Reg3a	Mm01181787_m1
Reg3b	Mm00440616_g1
Reg3g	Mm00441127_m1
Defa1	Mm02524428_g1
Defa-rs1	Mm00655850_m1
Defa5	Mm00651548_g1
Defa21	Mm04206099_gH
Muc1	Mm00449599_m1
Muc3	Mm01207064_m1
Muc13	Mm00495397_m1

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